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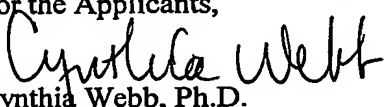
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**LOW-PRESSURE CHAMBER FOR SCANNING ELECTRON MICROSCOPY  
IN A WET ENVIRONMENT**

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**LOW-PRESSURE CHAMBER FOR SCANNING ELECTRON MICROSCOPY  
IN A WET ENVIRONMENT**

תא לחץ נמוך למיקרוסקופ אלקטרוני סורק להדמיה בסביבה רטובה

YEDA/016-IL

# **LOW-PRESSURE CHAMBER FOR SCANNING ELECTRON MICROSCOPY IN A WET ENVIRONMENT**

## **FIELD OF THE INVENTION**

5           The present invention relates to a low-pressure chamber for the electron microscopic examination of sample in a non-vacuum environment, wherein the sample is a wet sample.

## **BACKGROUND OF THE INVENTION**

10           In cell biology, as well as in the field of polymer science and in industries such as petroleum, food and microelectronics, microscopic observations in the range of few nanometers are highly desirable, and, in fact, the data obtained by such microscopic observations cannot be obtained by other techniques. Scanning electron microscopes (SEMs) have the ability to attain such high resolution, however, samples examined by  
15 SEMs have to be maintained in vacuum, precluding the study of in-vivo processes or the study of wet materials. In addition, electrically insulating samples composed of organic materials require coating to avoid charge accumulation; the preparation of samples for electron microscopy is time and labor consuming, and, furthermore raises concerns regarding the biological relevance of the results.

20           Attempts to maintain a living sample intended for electron microscopy have started as early as 1960 (a thesis by Thornley, University of Cambridge, 1960), and transmission electron microscopy (TEM) for examination of specimens at atmospheric pressure, for example in water, was described on 1970 (Swift and Brown J. Phys. E: Sci. Instrum. 3, 924, 1970). A cell having an aperture sealed with a collodion-carbon

film was used to mount a sample. An electron beam passes through the aperture to strike the sample, and electrons not stopped by the sample continue to a scintillator where photons are produced. At atmospheric pressure the results were found to be "rather noisy" although a resolution of 0.1 $\mu$ m was claimed.

5 US Patent Nos. 4,071,766 and 4,720,633 describe attempts to use electron microscopy to see material in a non-vacuum environment. In both of these patents the electron beam travels through an aperture to a wet specimen. Neither of these attempts succeeds, however, in successfully viewing wet objects. The contents of both of these documents are hereby incorporated by reference.

10 An Environmental Scanning Electron Microscope (ESEM), commercially available from Philips Electron Optics of Eindhoven, The Netherlands, is shown to maintain a vacuum gradient along the electron beam path, in an attempt to maintain a living sample. However, this ESEM require working with a sample at a critical point of water-vapor equilibrium, and cooling the sample to around 4°C. Inspection of  
15 specimens at pressures of up to 5 Torr is said to be possible. However, so far there is no evidence that wet and/or living objects can be viewed at resolutions of 10nm and below. Further information on this product and how it works can be found in US Patent Nos. 5,250,808, 5,362,964, and 5,412,211, the contents of which are hereby incorporated by reference.

20 A common method of achieving high-resolution inspection of organic matter is Transmission Electron Microscopy (TEM). TEM requires specially prepared specimens having typical thickness in the range of 50nm. A very high voltage is applied to create a parallel beam that passes through the sample. US Patent 5,406,087, the contents of which are hereby incorporated by reference, discloses a specimen holding device for use  
25 with a TEM. A specimen is sealed between two films that are able to transmit an

electron beam. The interior of the device is filled with moisture and may be placed within the vacuum environment of the TEM. A very high-energy beam travels through the specimen and surrounding fluid leading to a poor signal to noise ratio, as well as considerable damage to the sample.

5 Co-pending International Application PCT/IL01/01108 discloses a chamber adapted for use with a scanning electron microscope, where the chamber is designed to enable electron microscopy of wet samples. The chamber has at least one aperture sealed with a membrane, which is adapted to withstand a vacuum, and is transparent to electrons. By this configuration, the chamber is isolated from the vacuum, enabling the  
10 scanning of wet samples. The apparatus described in the aforementioned PCT application was a breakthrough prototype of a chamber suitable for environmental electron scanning microscopy (ESEM). However, the pressure gradient between the vacuum within the electron microscope and the approximately atmospheric pressure within the sample chamber creates some technical difficulties that remained only partly  
15 resolved.

Thus, there is a recognized need for, and it would be of great advantage to have an improved, more versatile chamber to meet with the diverse needs of scanning living samples.

## 20 **SUMMARY OF THE INVENTION**

It is an object of the present invention to provide a novel sample chamber adapted for use with a scanning electron microscope, which will enable electron microscopy of wet samples. The sample chamber has at least two apertures; a first aperture sealed by a partition membrane, which is adapted to withstand a vacuum and is transparent to  
25 electrons, and means for reducing the pressure difference across the partition

membrane, wherein a decreased pressure gradient enables the advantageous use of a thinner and lower density membrane for obtaining a better resolution. The means for reducing the pressure difference across the separating membrane further enable the versatile design of chambers for different specific applications.

5       The present invention provides a low-pressure chamber adapted for use within an electron microscope, preferably scanning electron microscope selected from an environmental scanning electron microscope (ESEM) or a low vacuum scanning electron microscope, comprising the following parts:

10       a sample chamber comprising at least two apertures; a first aperture sealed by a partition membrane adapted to withstand vacuum and transparent to electrons; and means for decreasing the pressure gradients across said partition membrane;

15       the means for decreasing the pressure gradient comprising at least a second open aperture in the sample chamber having a hollow member extending from said second open aperture of the sample chamber, said hollow member is open to the vacuum within the microscope.

20       The present invention further provides a modified low-pressure chamber adapted for use within an electron microscope, preferably scanning electron microscope selected from an environmental scanning electron microscope (ESEM) or a low vacuum scanning electron microscope, comprising the following parts:

25       the sample chamber described above;

      a liquid reservoir chamber, wherein the hollow member extending from said second aperture of the sample chamber is an elongated structure comprising a first end and a second end;

      the liquid reservoir chamber comprises at least two apertures, wherein the

elongated hollow member is connected at its first end to the second open aperture in the sample chamber and at its second end to the first of said apertures of the reservoir chamber;

a hollow member extending from a second open aperture of the reservoir chamber, said hollow member is open to the vacuum within the microscope.

The means for reducing the pressure gradient across the membrane comprise the open aperture and the hollow member opened to the vacuum within the microscope, designed to reach a water pressure in the sample while the microscope chamber pressure is kept lower by the microscope pump. The sample chamber pressure is regulated by the water itself, and is directly related to the sample temperature. In the modified low-pressure chamber, the water content in the sample chamber is regulated by the water vapor from the reservoir chamber.

Preferably, one open aperture is attached to an elongated member. In a preferred embodiment, the internal diameter of the aperture is substantially within the range of 50-100  $\mu\text{m}$  and the member extending therefrom is within the range of 1-100 mm in length. In one currently preferred embodiment, the diameter of the aperture is 100  $\mu\text{m}$  and the extending member is 100 mm in length.

In a preferred embodiment, the sample and the reservoir chambers are adapted to hold water or any aqueous medium, wherein the air water surface in the reservoir chamber is much larger compared to the air-water surface in the sample chamber. In one currently preferred embodiment the air-water surface is 2 to 10 times larger in the reservoir chamber compared to the sample chamber. The reservoir chamber may be located inside or outside the microscope chamber.

In one currently preferred embodiment, the reservoir chamber is located inside the microscope chamber.



In yet further currently preferred embodiment, the aqueous medium in the sample chamber is a growth medium according to the sample type, and the aqueous medium in the reservoir chamber is pure (doubled distilled) water.

Preferably, the membrane sealing the sample chamber apertures is resistant to  
5 pressure gradient of 10-80 mbar, having a thickness lower than 150 nm.

In a preferred embodiment, the membrane is selected from the group consisting of polyimide, polyamide, polyamide-imide, polyethylene and polypyrrole membranes or films known in the art to support samples in TEM experiments.

In yet currently preferred embodiment, the membrane is selected from, but not  
10 restricted to pure FormVar™ membranes; FormVar™ membranes covered with Carbon and pure Carbon films. In one currently most preferred embodiment the membrane selected for sealing the patent aperture of the sample chamber is pure FormVar™ (30-60 nm thick).

This membrane is thinner compared to the membranes described previously (co-  
15 pending International Application No. PCT/IL01/01108), those were at a range of 50-150 nm thick. Pure FormVar™ is a material with lower density compared to the previously disclosed polyimide. The use of such membrane is highly advantageous as thinner and lower-density membranes give better resolution imaging and higher signal intensity.

20 Preferably, the membrane is set on a support, and a TEM grid is placed on the counter side of the membrane. Said "unit" comprising the support, the membrane and the grid is placed on a ring, and a sample is placed in proximity to the membrane.

In a preferred embodiment a thin membrane is mounted directly on a TEM grid,  
the grid is set on a ring, and the sample is placed with a direct contact with the  
25 membrane.

In yet another embodiment, an inverted sample chamber is disclosed, enabling an observation of the counter-side of a sample. In the inverted chamber, the grid and the membrane are placed on the same side of the support, and placed on a ring with the sample facing the electron beam. A curved surface that enables undisruptive press of the  
5 membrane to the sample should be created. Different methods may be employed to prepare said curved surfaces including, but not limited to, the use of glass slides covered with UV glue drop or flamed silicon oil.

In a preferred embodiment, few small canals are made in the ring on which the sample is placed to allow the excess liquid between the glass and the membrane to drip  
10 into the chamber.

In general, the low-pressure chamber disclosed in this invention is placed in the microscope chamber. The pressure in the microscope chamber is 0.0-9.0 Torr, and the pressure in the sample chamber is the vapor pressure of the aqueous solution in which the sample is placed, said vapor pressure depends on the temperature of the aqueous  
15 solution.

In a preferred embodiment, the pressure in the microscope chamber is 0.1 Torr and the temperature in the sample chamber is the optimal sample temperature, for example 37°C for cell-culture samples, wherein the water vapor pressure is 62.7 mbar.

In another aspect the present invention provides additional set up of a sample  
20 chamber with different compartments. The compartments are not isolated from each other, enabling air exchange between samples.

Preferably, the different compartments are different wells in a well plate, said well plate is fixed on a support, said sealing membrane is placed on the plate and covered by said grid.

25 In one currently preferred embodiment, the sealing membrane is a polyimide

membrane.

In yet another aspect, the present invention provides fluid-exchangeable sample chamber, wherein fluid exchange is enabled by insertion of two channels in the direction of the center of the ring supporting the membrane. This modification is made possible by the innovative design of the present invention, wherein the chamber is opened to the microscope chamber, and wherein a change of the liquid in the sample chamber does not affect the pressure. The possibility to modify the medium in the sample chamber while the sample is under observation is highly advantageous, enabling the examination of different conditions with the same sample.

According to yet another aspect the present invention provides a method of observing wet samples at high resolution, comprising the steps of placing a wet sample in a chamber, wherein said chamber is opened to a scanning electron microscope chamber through an aperture sealed with a barrier and through a second opened aperture, providing an electron beam to strike said sample from said microscope chamber through said barrier, and observing at least one of secondary and backscattered electrons emerging from said chamber.

Preferably, the barrier comprises a membrane selected to withstand a pressure gradient substantially lying at a range of 10-80 mbars and to be transparent to electrons having energy in excess of around 2keV.

Preferably, the wet sample comprises a pharmaceutical composition.

Preferably, the wet sample further comprises a living cell with which the pharmaceutical composition is interacting.

Preferably, said chamber is a fluid exchangeable chamber wherein the pharmaceutical composition is changed in respect of one of a group comprising concentration and type dynamically during the observation.

Preferably, said chamber comprises different compartments wherein each compartment may contain different pharmaceutical composition.

### **BRIEF DESCRIPTION OF THE FIGURES**

5 FIG. 1: The advantage of a thin and lower density membrane: better resolution imaging and higher signal intensity

FIG.2: A. Basic low-pressure chamber set up

B. Modified low-pressure chamber set up, including the reservoir chamber

10 FIG. 3: Different possible low-pressure chamber designs

FIG 4: A diagram exemplifying a low-pressure chamber set up.

A. Top view

B. Cross section

FIG 5: Low-pressure chamber designed for different compartments

15 FIG.6: Fluid exchangeable low-pressure chamber

FIG 7: Inverted sample set up

FIG 8: A. 20 nm-gold particles observed by ESEM equipped with low-pressure chamber

20 B. Detection of 10 nm colloidal gold by ESEM equipped with low-pressure chamber

### **DETAILED DESCRIPTION OF THE INVENTION**

The present invention provides a novel sample chamber adapted for use with a scanning electron microscope, which will enable electron microscopy of wet samples.

25 The present invention is based on a sample chamber wherein a wet environment is

provided in a small chamber sealed by a membrane that is thin enough for energetic electrons to pass through and interact with the sample being studied.

In the present invention we now disclose a sample chamber with means for reducing the pressure difference across the partition membrane, wherein a decreased  
5 pressure gradient enables the advantageous use of thinner and lower density membranes. The use of said membranes results in a better resolution imaging and higher signal intensity as exemplified herein below. In addition, the reduced pressure allows the versatile design of the chamber for adaptation to various applications such as multi-compartment chambers, fluid exchangeable chambers, reverse chambers etc., as is  
10 also exemplified herein below.

The present invention provides a low-pressure chamber adapted for use within an electron microscope, preferably with an electron microscope selected from an environmental scanning electron microscope (ESEM) or a low vacuum scanning electron microscope, comprising the following parts:

15 a sample chamber comprising at least two apertures; a first aperture sealed by a partition membrane adapted to withstand vacuum and transparent to electrons; and means for decreasing the pressure gradients across said partition membrane;  
the means for decreasing the pressure gradient comprising at least a second  
20 open aperture in the sample chamber having a hollow member extending from said second open aperture of the sample chamber, said hollow member is open to the vacuum within the microscope.

The present invention further provides a modified low-pressure chamber adapted for use within an electron microscope, preferably scanning electron microscope selected  
25 from an environmental scanning electron microscope (ESEM) or a low vacuum

scanning electron microscope, comprising the following parts:

the sample chamber described above;

a liquid reservoir chamber, wherein the hollow member extending from said second aperture of the sample chamber is an elongated structure comprising a first end and a second end;

the liquid reservoir chamber comprises at least two apertures, wherein the elongated hollow member is connected at its first end to the second open aperture in the sample chamber and at its second end to the first of said apertures of the reservoir chamber;

a hollow member extending from a second open aperture of the reservoir chamber, said hollow member is open to the vacuum within the microscope.

The basic set up of the low-pressure chamber is illustrated in Fig. 2A and B: [2] sample chamber, [4] open aperture, [6] hollow member, [8] reservoir chamber, and [10] membrane.

The chamber design of the present invention is based on one of its physical features, wherein the pressure gradient across the membrane is given by the following term:

$$(1) P_{\text{wet chamber}} - P_{\text{microscope chamber}}$$

wherein the pressure in each compartment depends on the microscope set up in use.

In the ESEM,  $P_{\text{microscope chamber}}$  can be varied from 0 to more than 10 Torr, preferably being at 4.5-8 Torr, a pressure rate considered as the optimal pressure for observation in the ESEM mode (high SE signal amplification, low beam scattering).

According to the present invention, the pressure within the microscope chamber can be further reduced, to reach a range of 0.0-4.5 Torr.

$P_{\text{wet chamber}}$  is the vapor pressure of the aqueous solution in which the sample is

placed, that is directly related to the aqueous solution temperature.

In a preferred embodiment of the present invention, the means for reducing the pressure gradient across the membrane of the sample chamber comprise a second open aperture of the sample chamber and a hollow member attached to it, wherein the hollow member is opened to the vacuum within the microscope. Reducing the pressure within the microscope chamber by the microscope pump result in a reduction of the pressure in the sample chamber, leading to water evaporation from the wet sample. However, the small open second aperture limits the rate of water vapor transfer into the microscope chamber, thus the pressure within the microscope chamber is kept constant by the microscope pump. The sample chamber pressure is regulated by the vapor pressure of the aqueous solution in which the sample is kept, and is directly related to the sample temperature.

In a preferred embodiment, a modified low-pressure chamber is disclosed, wherein the hollow member extended from the second open aperture in the sample chamber is attached at its second end to a reservoir chamber, adapted to contain aqueous solution. This set up further reduces water evaporation from the sample, as water evaporates from the two liquid-vapor interfaces at the same time. The open second aperture of the reservoir chamber, to which a hollow member is attached, regulates water vapor into the microscope chamber.

In yet further preferred embodiment, evaporation from the reservoir chamber is increased relatively to the evaporation from the sample chamber, by means selected from the group consisting of the geometry of the apparatus, the liquid-vapor interface area, the respective temperature of each chamber and the osmotic potential of each aqueous solution.

In a preferred embodiment, the geometry of the low-pressure chamber is designed

for maximum reduction of the evaporation from the sample. In said geometry, the open second aperture of the sample chamber is located as far as possible from the sample itself, and the hollow member connects the sample chamber to the reservoir chamber such that the reservoir chamber would be in between the sample itself and the sample chamber aperture. The reservoir chamber, on a microscope stage [12] may be located within (Fig. 3A) or outside (Fig.3B) the microscope chamber. In a further preferred embodiment, the open aperture of the reservoir chamber is attached to a hollow elongated member, opened to the microscope chamber. Preferably, the internal diameter of the aperture is 50-100 $\mu$ m and the member extending therefrom is 1-100 mm in length. In one currently preferred embodiment, the diameter of the aperture is 100 $\mu$ m and the extending member is 100mm in length.

In yet further currently preferred embodiment, the air-water surface is 2 to 10 times higher in the reservoir chamber compared to the sample chamber and the aqueous medium in the sample chamber is a growth medium according to the sample type, while the aqueous medium in the reservoir chamber is pure (doubled distilled) water.

The present invention discloses the use of thin, low-density membranes as a partition membrane sealing one of the sample chamber apertures, isolating the wet sample from the vacuum within the microscope chamber. The use of said membranes increases the resolution obtained by the scanning electron microscope, and, by enhancing the signal amplitude, reduces the threshold for sample detection.

In a preferred embodiment, the membrane sealing the sample chamber apertures is selected from the group consisting of polyimide, polyamide, polyamide-imide, polyethylene and polypyrrole membranes or films known in the art to support samples in TEM experiments.

In a more preferred embodiment, the membrane is selected from, but not restricted



to pure FormVar™ membranes; FormVar™ membranes covered with Carbon and pure Carbon films.

In one currently most preferred embodiment the membrane selected for sealing the patent aperture of the sample chamber is pure FormVar™ (30-60 nm thick).

5 In another aspect, the present invention discloses the geometry set up of the low-pressure chamber. The set up is based on the principles described herein above; however, it is known to one skilled in the art that the set up described herein bellow is given as a non-limiting example only. Any other set up that would answer the basic features of the low-pressure chamber may be also used.

10 The present invention discloses the modified low-pressure chamber set up (Fig. 4) wherein the sealing membrane is set on a support [14], and a TEM grid [16] is placed on the counter side of the membrane. Said "unit" comprising the support, the membrane and the grid is placed on a ring [18] (Fig. 4A, I) and a sample [24] is placed in proximity to the membrane. The ring is placed such that the membrane seal one aperture  
15 of the sample chamber and is set in place by an "O"-ring [20] and by frame [22]. The second aperture is connected to a hollow member that its other side is connected to a first aperture in the reservoir chamber. The second small aperture in the reservoir chamber is attached to a hollow member, said hollow member is opened into the vacuum within the microscope chamber.

20 In a preferred embodiment a thin membrane is mounted directly on a TEM grid, the grid is set on a ring, and the sample is placed with a direct contact with the membrane (Fig. 4B, II).

In yet further currently preferred embodiment the reservoir chamber is placed underneath the sample chamber, wherein the second open aperture of the sample  
25 chamber extends to the first open aperture of the reservoir chamber. The second small

hollow member opened to the vacuum within the microscope is made of a syringe tip having an internal diameter of 100 $\mu$ m as exemplified in Fig. 4 herein bellow. The aqueous solution in the sample chamber is a medium or a buffer adapted to the cells, and the aqueous solution in the reservoir chamber is pure (double distilled) water.

5        The low-pressure chamber comprising the set up described above is placed within the chamber of the electron scanning microscope, preferably a scanning electron microscope selected from the group consisting of ESEM or low-vacuum scanning microscope.

10        In yet another aspect, the present invention discloses low-pressure chambers adapted to various applications.

In one preferred embodiment, the present invention discloses a single chamber with different compartments. The compartments are not isolated from each other thus permitting air exchange among the different compartments.

15        The geometric set up design of the multi compartment chamber is basically the same as the one-compartment sample chamber, except that a well plate [26] is located between the support and the membrane and the parts are closed together by screws [28], as exemplified in Fig. 5 herein bellow.

In one currently preferred embodiment, the membrane used for the multi compartment chamber is polyimide membrane.

20        In yet further preferred embodiment, the present invention discloses a fluid exchangeable sample chamber. This adaptation of the sample chamber is made possible by the innovative design of the present invention, wherein the low-pressure chamber is opened to the microscope chamber and therefore a change of the sample aqueous solution does not affect the pressure. The possibility to modify the medium in the  
25        sample chamber while the sample is under observation is highly advantageous, enabling

the examination of different conditions with the same sample.

In one currently preferred embodiment, a slight modification in the set up of the basic low-pressure chamber is made in order to enable fluid exchange, wherein two channels [30] in the direction of the chamber center are curved in the ring supporting the membrane. Two syringe tips [32] are inserted into the channels, and fluid may be injected or pumped through the syringe as exemplified in Fig. 6 herein bellow.

In yet another preferred embodiment, an inverted low-pressure chamber is designed to enable visualizing the counter-side of a sample.

In one currently preferred embodiment, the geometric set up of the inverted sample comprises a grid and a membrane set on the same side of the support. The resulted "unit" is placed on a ring with the sample facing the electron beam, as exemplified in Fig. 7 herein bellow. In order to make a good contact between the sample and the membrane, a curved surface should be placed in the interface between the sample and the membrane, wherein pressing the sample to the membrane will not be destructive.

In one currently preferred embodiment, the curved surface is selected from the group consisting of glass slides covered with UV glue drop or flamed silicon oil.

According to yet another aspect the present invention provides a method of observing wet objects at high resolution, comprising the steps of placing a wet object in a chamber, wherein said chamber is opened to a scanning electron microscope, preferably to an ESEM or low vacuum microscope chamber through an open aperture, and through an aperture sealed with a barrier, providing an electron beam to strike said sample from said microscope chamber through said barrier, and observing at least one of secondary and backscattered electrons emerging from said chamber.

In a preferred embodiment, the barrier comprises a membrane selected to

withstand a pressure gradient lying substantially at the range of 10-80 mbars, to be transparent to electrons having energy in excess of around 2keV, and to be compatible for cell growth.

5 In yet another preferred embodiment, the patent aperture of the sample chamber is attached to a hollow member, said hollow member is attached at its other side to an aperture in the reservoir chamber, and water vapor is injected into the microscope chamber through a second thin hollow member attached to a second aperture in the reservoir chamber.

Preferably, the wet sample comprises a pharmaceutical composition.

10 Preferably, the wet sample further comprises a living cell with which the pharmaceutical composition is interacting.

In yet further preferred embodiment, said chamber is a fluid exchangeable chamber, wherein the pharmaceutical composition is changed in respect of one of a group comprising concentration and type dynamically during the observation.

15 Preferably, said chamber comprises different compartments wherein each compartment may contain different pharmaceutical composition.

The principles of the invention, using a low-pressure chamber for electron microscopy with SEM and low-vacuum scanning microscope, according to the present invention, may be better understood with reference to the following non-limiting

20 examples.

## **EXAMPLES**

### **Example 1: Thinner and lower density membrane give better resolution imaging and higher signal intensity**

We have previously shown that the imaging resolution of small gold particles, obtained by scanning electron microscope is related to the size of the electron beam when it reaches the gold particles. Before reaching the gold particles, the beam is scattered by the medium in which it is traveled, comprising the separating membrane and a water layer if the particle is not in direct contact with the membrane. In a wet sample, the water layer always exists, and its width cannot be controlled. However, it is possible to control the membrane type, and by reducing its width to reduce the membrane influence on the beam scattering. Not only that thin membrane improves the resolution, it will also enhance the signal amplitude thus improving the ability to detect objects of low signal intensity like small gold particles. This phenomenon can be explained by the following example:

Consider a gold particle located just below the membrane, wherein the membrane is thick and dense compared to a thin and light membrane. The beam traveling through the thin membrane is less scattered, and therefore gives a narrower pick signal. Under both conditions the integrated signal -the pick surface- would be the same; however, when the membrane is thin and light the signal is narrower and higher (Fig1.b) compared to the signal obtained when the membrane is thick and dense (Fig.1a), thus enables better detection. As shown in Fig 1c the signal coming of a thin membrane is further enhanced as the back-scattered electrons have a higher probability to reach the detector without encountering another scattering event in the membrane.

### **Example 2: Basic set up for the low-pressure chamber**

We have assembled two basic set ups for the low-pressure chamber: one without (Fig. 2A) and one with (Fig. 2B) a reservoir chamber.

5 Few possible members were examined to form a patent aperture with a hollow member attached to it. Syringe tips with internal diameter of 100  $\mu\text{m}$  and 100 mm length were examined. The syringe with the tip was filled with a certain amount of water, and placed inside a vacuum chamber for few hours. The syringe was weighed before and after it was placed in the vacuum chamber. The weight difference gave the amount of water evaporated from the aperture.

10 Six independent measurements showed that the evaporation rate from this kind of aperture is 50-60  $\mu\text{l}$  per hour. Changing the internal diameter of the aperture and the length of the hollow member may change this rate.

When the set up included a reservoir chamber, the excess amount of water within this chamber provided water vapor that kept the sample chamber wet for few hours.

### **15 Example 3: Examining the mechanical properties of a membrane**

We developed a general method for examining the membrane resistance to pressure.

20 The membrane (together with a 400 Mesh grid) was attached to one opening of a hollow column filled with water. The second opening of the column was lifted progressively to a position higher than that of the membrane, thus creating pressure on the membrane. A column with 10 cm of water gives a pressure of 9.81 mbar on the membrane. Few membrane types were examined; FormVar™ membrane was found to be resistant to a column of 80 cm, creating a higher pressure than that anticipated in the low-pressure chamber.

#### **Example 4: Basic protocol for preparing a sample for observation**

An aqueous solution is suitable for use as a sample medium; the specific medium should be selected in accordance with the sample type. However, when introducing a living cell within the sample to be scanned, bicarbonate buffer should be avoided.

- 5 Bicarbonate buffers release  $\text{CO}_2$  to the atmosphere, resulting in accumulation of  $\text{OH}^-$  ions in the sample and pH elevation. Such pH elevation may cause cell death.

We have tested media based on HEPES for the growth of NIH3T3 cells at  $37^\circ\text{C}$  and the corresponding vapor pressure for few days. A normal cell growth over the time was observed.

#### 10 **Example 5: Growing cells on thin membrane**

Three kinds of membranes were tested:

FormVar™ (30-75nm thick)

FormVar™-Carbon (30 to 70nm thick)

Carbon (20 to 30nm thick)

- 15 The membranes were first ion discharged for about a minute, and then incubated with poly-L-Lysine at 0.1mg/ml overnight, washed 3 times for 5 minutes with water and one time with PBS. Optionally, the membrane was then incubated with Fibronectin (0.1% weight) for 1 hour, and washed again 3 times for 5 minutes with PBS. Cells were grown in suitable medium.

- 20 Our tests showed good cell growth on the 3 membrane types examined. The only problem appeared when Carbon membranes were used, if the grid used was made of Copper. It seemed that the Copper, which is a toxic substance, diffused through the Carbon membranes and harmed the growing cells. The two other membranes – FormVar™ alone and FormVar™-carbon, did not cause any harm to the cells even

when copper grids were used. With carbon membrane it would therefore be more efficient to use nickel or gold grids.

We succeed to grow the cells on carbon membranes, meaning that they are not porous to water as far as we can experimentally noticed. However, after long exposure to water (ten hours and above), carbon membranes were found to be more sensitive to damages than other membranes.

#### **Example 6: Observations with the low-pressure chamber**

Gold particles of 20nm attached to protein A in PBS were imaged at room temperature (24°C) by ESEM equipped with the low-pressure chamber of the present invention. The membrane used was a pure carbon membrane with copper 400 mesh grid (Electron Microscopy Science). The designated thickness of the membrane was 20-30nm. The microscope was in a low vacuum mode (maximum pressure of 1.5 Torr – low vacuum aperture).

As shown in Fig. 8A the resolution obtained was higher than 20nm, as two beads in contact could be resolved. As shown in Fig. 8B, even 10nm particles (colloidal gold) could be detected. The slightly elongated shape of the beads is due to slow charging of the sample during the experiment. Connecting the sample to the ground can solve this problem.

During the course of the experiment, after few hour of exposure to water the carbon membrane was slightly damaged.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying current knowledge, readily modify and/or adapt for various applications such specific embodiments without undue



experimentation and without departing from the generic concept, and, therefore, such adaptations and modifications should and are intended to be comprehended within the meaning and range of equivalents of the disclosed embodiments. It is to be understood that the phraseology or terminology employed herein is for the purpose of description and not of limitation. The means, materials, and steps for carrying out various disclosed chemical structures and functions may take a variety of alternative forms without departing from the invention. Thus the expressions "means to..." and "means for...", or any method step language, as may be found in the specification above and/or in the claims below, followed by a functional statement, are intended to define and cover whatever chemical structure, or whatever function, which may now or in the future exist which carries out the recited function, whether or not precisely equivalent to the embodiment or embodiments disclosed in the specification above, i.e., other means or steps for carrying out the same functions can be used; and it is intended that such expressions be given their broadest interpretation.

15

## CLAIMS

1. A low-pressure chamber suitable for use in a scanning electron microscope,  
said chamber comprising:  
a sample chamber comprising at least two apertures;  
5 a first aperture sealed by a partition membrane and means for decreasing the  
pressure gradient across said partition membrane;  
the means for decreasing the pressure gradient comprising at least a second  
open aperture in the sample chamber having a hollow member extending  
from said second open aperture of the sample chamber.
- 10 2. The low-pressure chamber of claim 1 comprising the sample chamber further  
comprising a liquid reservoir chamber.
3. The low pressure chamber of claim 2, wherein the hollow member extending  
from said second aperture of the sample chamber is an elongated structure  
comprising a first end and a second end;  
15 the liquid reservoir chamber comprises at least two apertures, wherein the  
elongated hollow member is connected at its first end to the second open  
aperture in the sample chamber and at its second end to the first of said  
apertures of the reservoir chamber;  
a hollow member extending from a second open aperture of the reservoir  
20 chamber.
4. The low-pressure chamber according to any one of claims 1-3 adapted to use  
within an electron-scanning microscope selected from environmental  
electron scanning microscope (ESEM) or low-vacuum microscope.
- 25 5. The low-pressure chamber of any one of claims 1-4 wherein the hollow  
member has one end open to the vacuum within the microscope, said open

end having an internal diameter within the range of 50-100  $\mu\text{m}$ .

6. The sample chamber according to any one of claim 1-4 wherein the sealing membrane thickness is less than 150 nm.
7. The sample chamber according to any one of claim 1-4 wherein the sealing membrane is resistant to a pressure lying substantially within a range of 10-80 mbars and is transparent to electrons having energy of at least 2keV.
8. The sample chamber according to any one of claim 1-4 wherein the sealing membrane is selected from the group consisting of polyimide, polyamide, polyamide-imide, polyethylene and polypyrrole membranes or films.
9. The sample chamber according to claim 8 wherein the sealing membrane is selected from the group consisting of pure FormVar™ membranes, FormVar™ membranes covered with Carbon and pure Carbon films.
10. The sample chamber according to claim 9 wherein the sealing membrane is pure FormVar™.
11. The low-pressure chamber according to any one of claims 1-4 wherein the sample chamber and the reservoir chamber are adapted to hold water or aqueous solutions.
12. The low-pressure chamber according to claim 11 wherein the air-water surface of the reservoir chamber is larger than the air-water surface of the sample chamber.
13. The low-pressure chamber according to claim 12 wherein the air-water surface of the reservoir chamber is 2 to 10 times larger than the air-water surface of the sample chamber.
14. The low-pressure chamber according to claim 11 wherein the medium in the sample chamber is aqueous solution and the medium in the reservoir

chamber is pure water.

15. The sample chamber according to any one of claim 1-4 comprising a support, wherein said membrane is set on said support.
16. The sample chamber according to claim 15 further comprising a grid,  
5 wherein said grid is set on the counter side of said membrane; wherein the membrane, the support and the grid compose a "unit".
17. The sample chamber according to claim 16 further comprising a ring, wherein said "unit" is placed on said ring.
18. The sample chamber according to claim 17, wherein a sample is placed in  
10 proximity with said membrane.
19. The sample chamber according to claim 18, wherein the sample is placed in a direct contact with said membrane, and said support is absent.
20. The sample chamber according to any one of claims 18-19 further comprising a frame.
- 15 21. The low-pressure chamber according to claim 2 wherein the sample chambers is placed within the scanning-electron microscope and the reservoir chamber is placed outside the scanning-electron microscope.
22. A sample chamber according to claim 17 for observation of the counter-side  
20 of a sample, wherein said supporting grid is set on the same side of said support to form an inverted sample chamber, wherein said unit is fixed to said ring with said sample facing the electron beam.
23. The inverted sample chamber according to claim 22 further comprising a curved surface.
24. The inverted sample chamber according to claim 23, wherein said curved  
25 surface comprises glass slide covered with glue.

25. The inverted sample chamber according to claim 23, wherein said curved surface comprises glass slide covered with flamed silicon oil.
26. A sample chamber according to any one of claim 1-4 for multi-sample measurements, comprising a well plate to form multi-compartment sample chamber.
27. The multi-compartment sample chamber of claim 26, wherein said membrane is placed on said well plate.
28. The multi-compartment sample chamber of claim 27 further comprising a support.
29. The multi-compartment sample chamber of claim 28 further comprising a grid, wherein said grid is placed on said membrane, wherein the support, the well plate, the membrane and the grid compose a multi-compartment unit.
30. A fluid exchangeable sample chamber according to claim 17, further comprising two curved channels within said ring.
31. The fluid exchangeable sample chamber according to claim 30 further comprising two hollow members, wherein each hollow member is inserted in each said channels.
32. A method for changing a fluid within a fluid exchangeable sample chamber comprising the steps of pumping out a fluid through one said hollow member and introducing a fluid through second said hollow member.
33. A method of observing wet samples at high resolution comprising the steps of placing a wet sample in a chamber, wherein said chamber is opened to a scanning electron microscope chamber through a patent aperture, and through an aperture sealed with a barrier, providing an electron beam to strike said sample from microscope chamber through said barrier, and

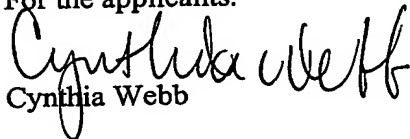
observing at least one secondary and backscattered electrons emerging from  
said chamber.

34. The method according to claim 33 wherein the barrier comprises a  
membrane selected to withstand a pressure substantially lying at a range of  
10-80 mbars and to be transparent to electrons having energy of at least  
2keV.

35. The method of claim 34 wherein the sample comprises a pharmaceutical  
composition.

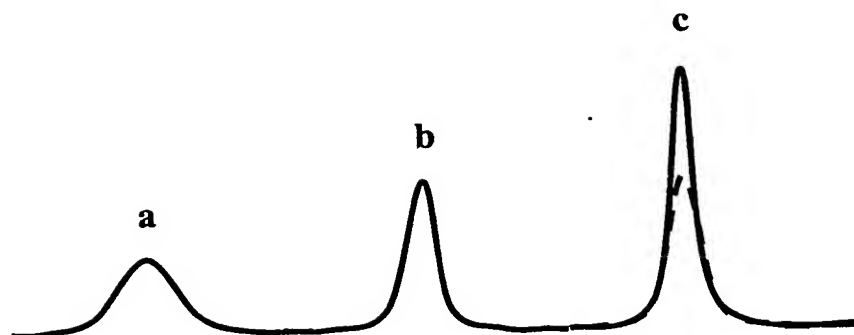
36. The method of claim 35 wherein the wet sample further comprises living  
cells with which the pharmaceutical composition is interacting.

For the applicants:

15   
Cynthia Webb

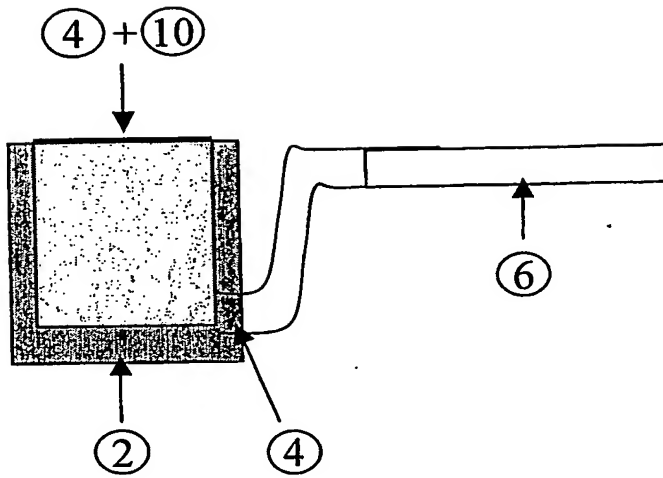
Webb & Associates

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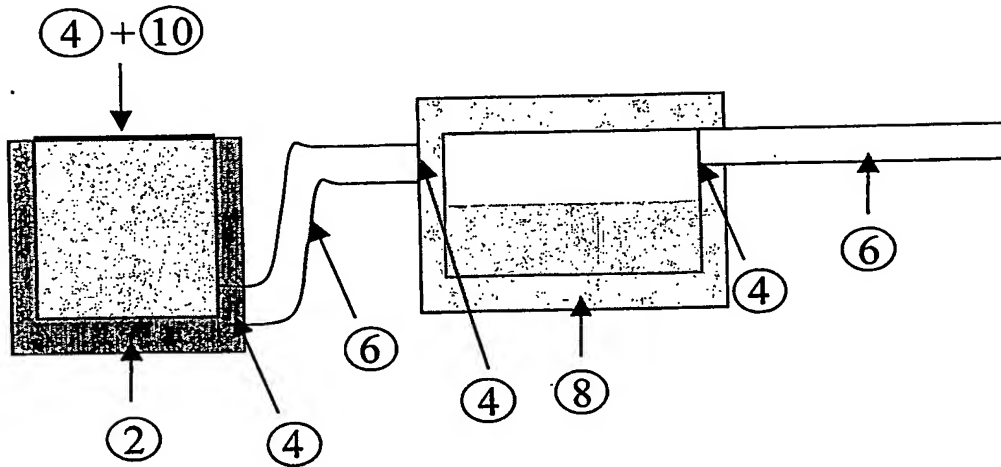


**FIG 1**

**A**



**B**

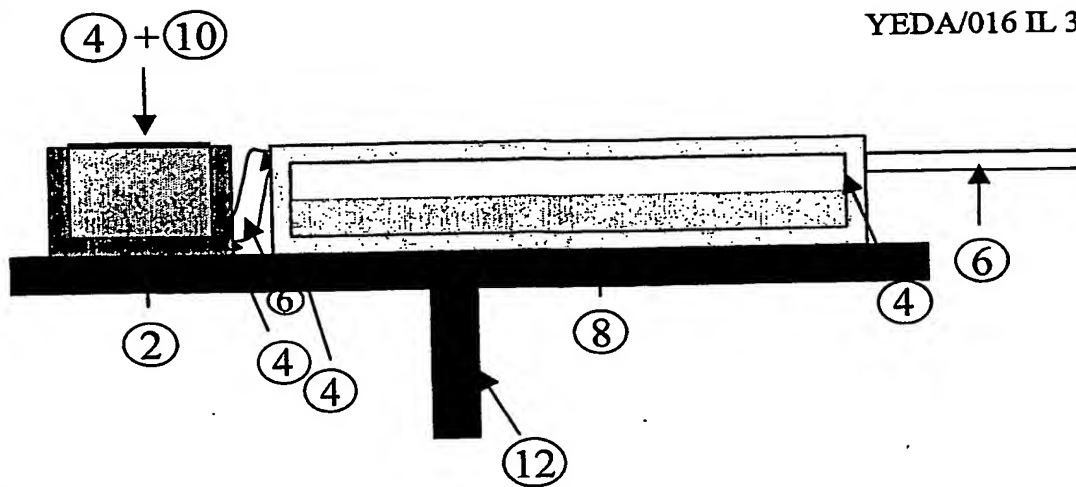


**FIG 2**

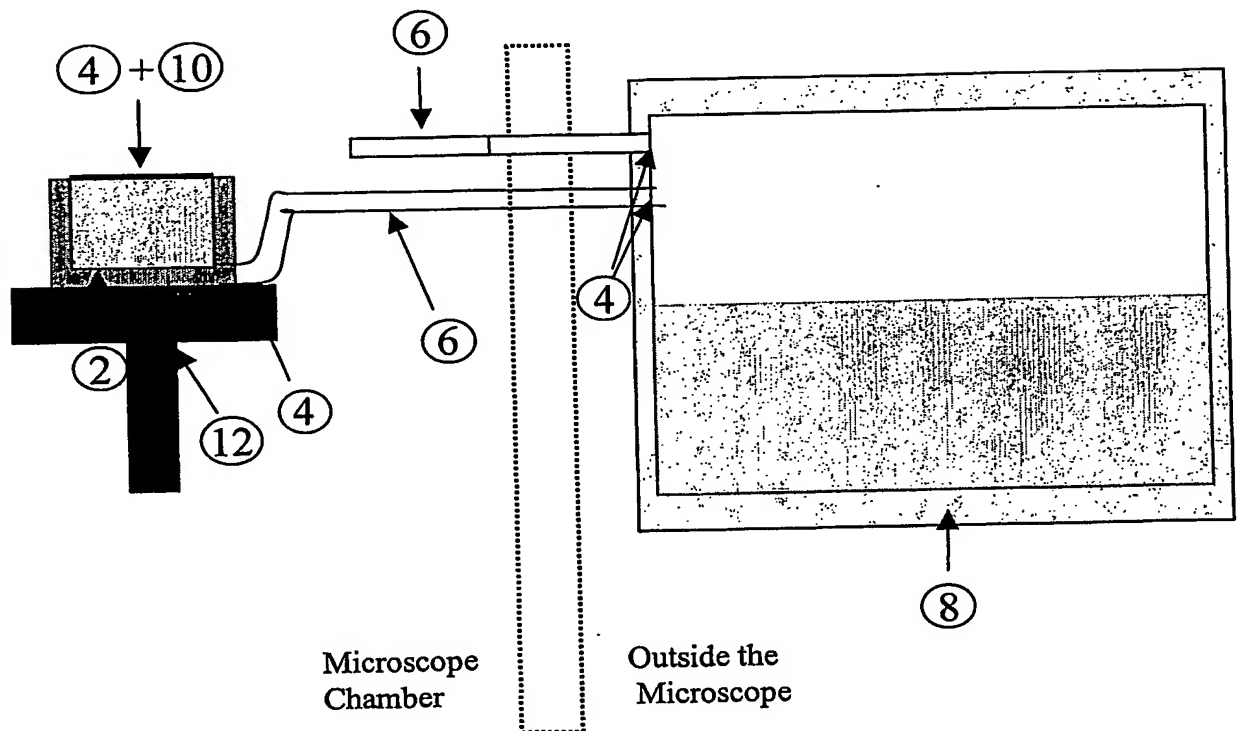


**A**

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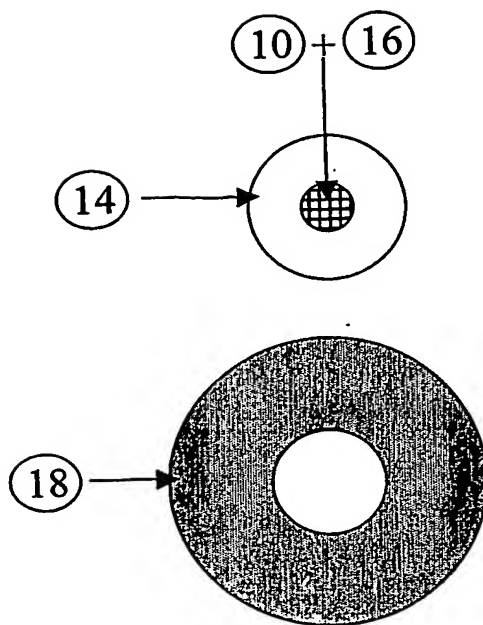


**B**

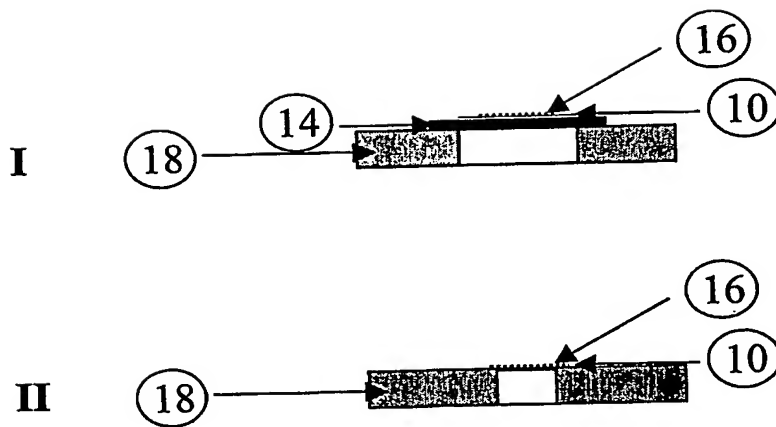


**FIG 3**

**A**

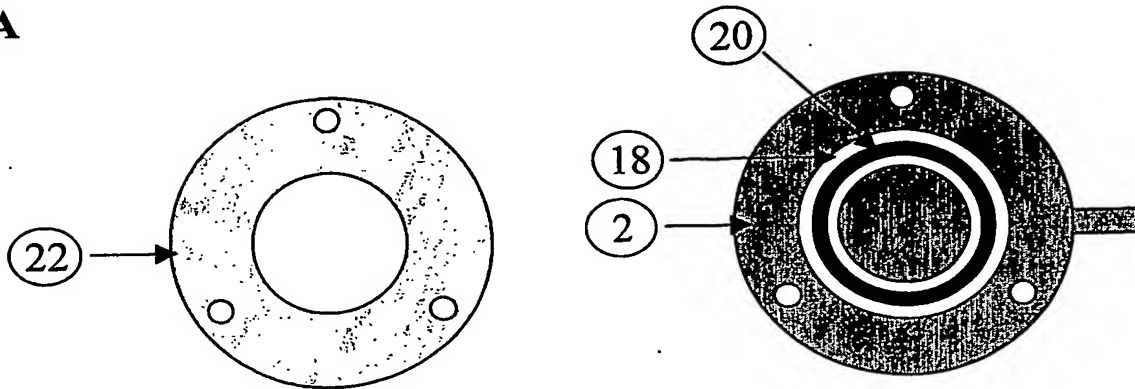


**B**

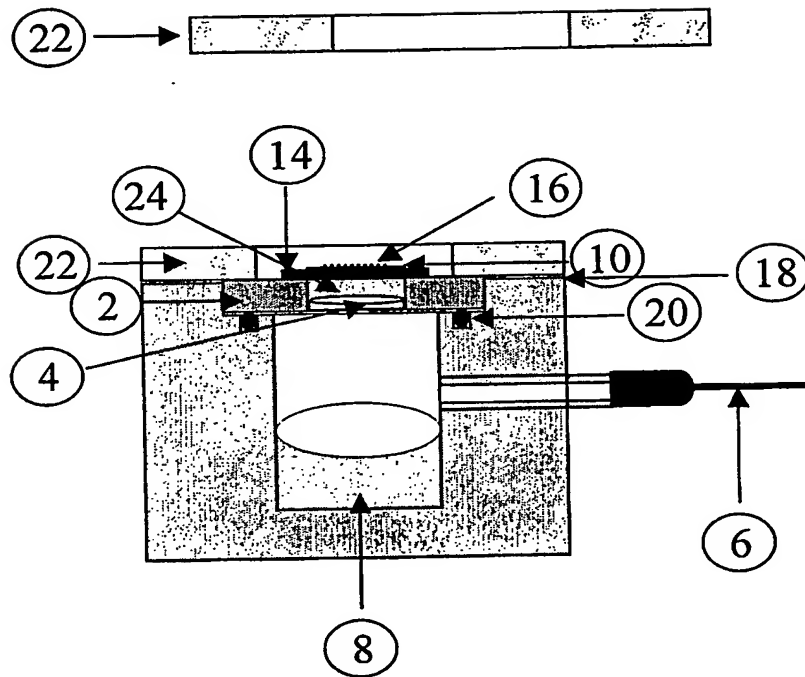


**FIG 4**

**A**



**B**



**FIG 4 (Continued)**

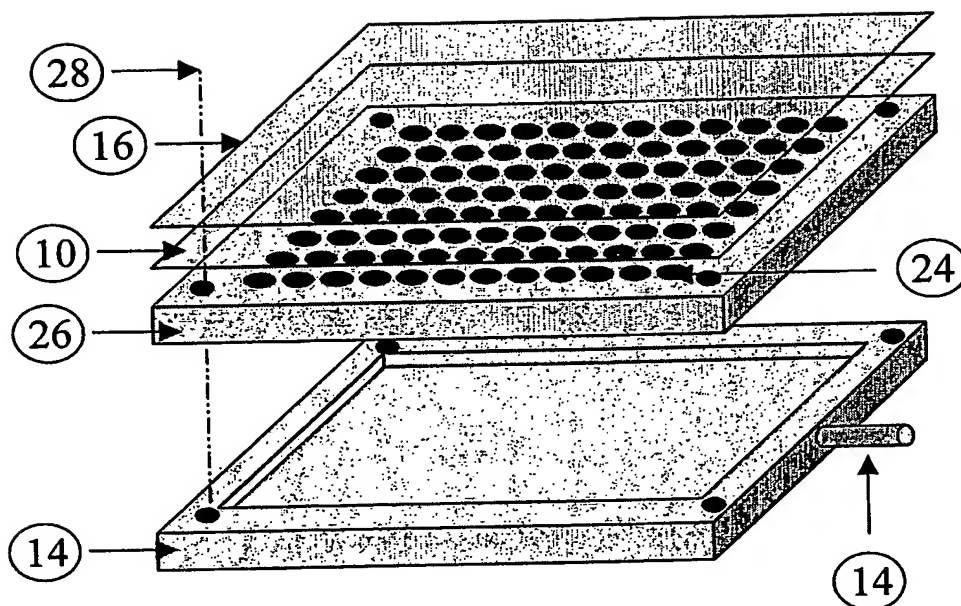


FIG 5

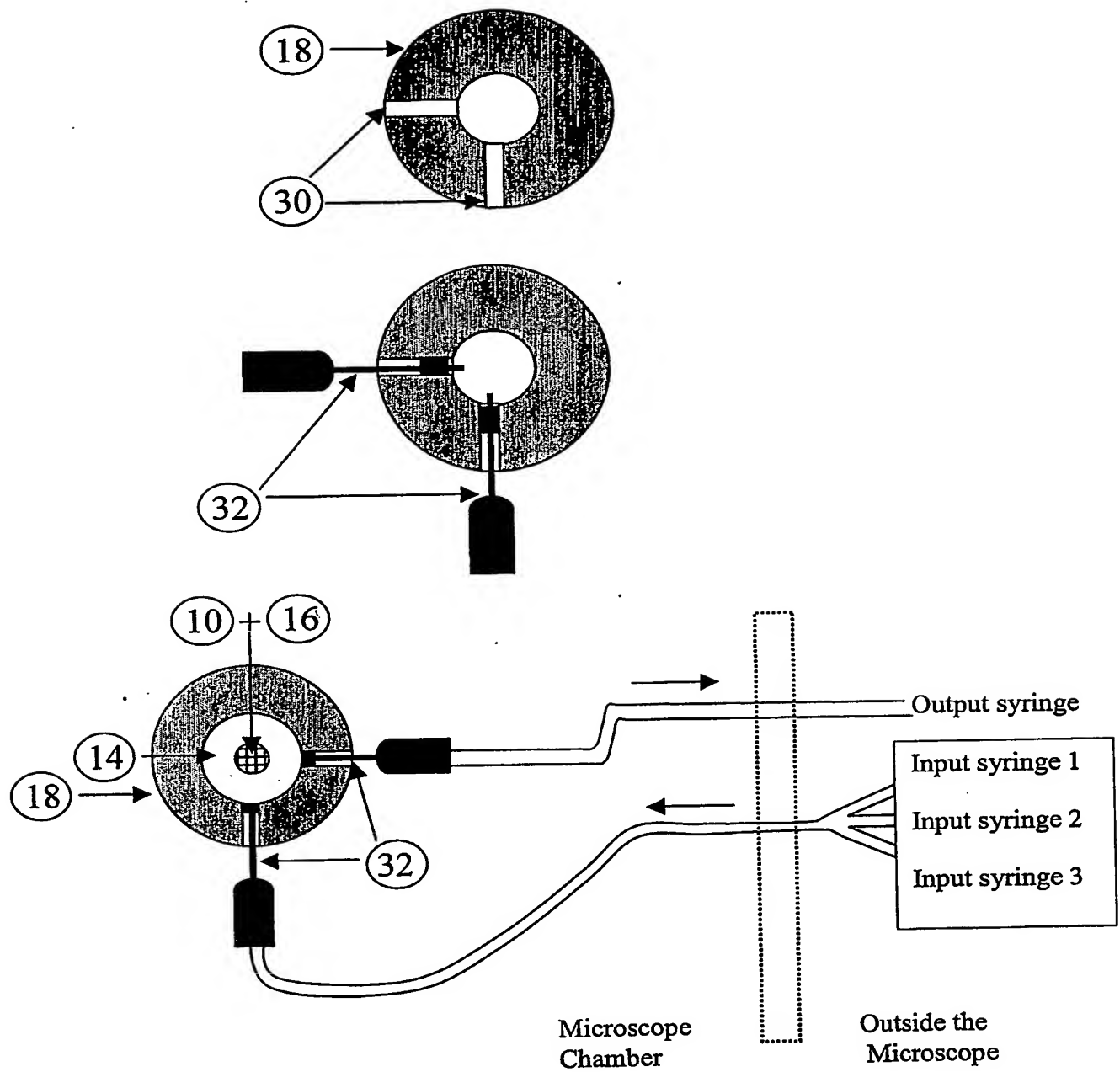
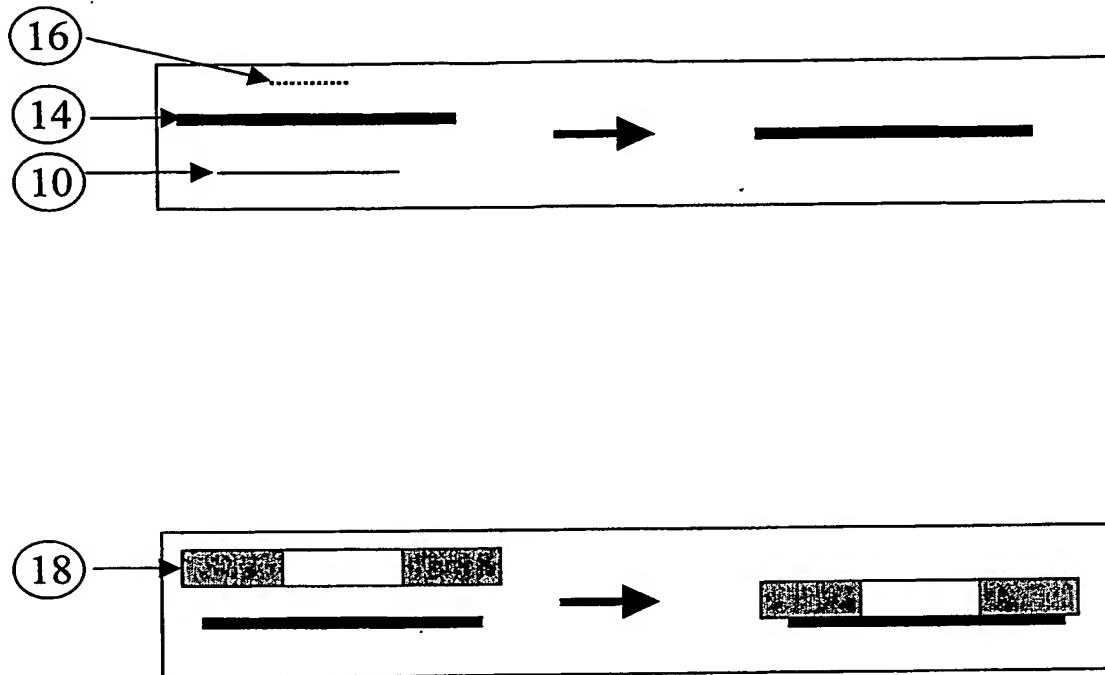


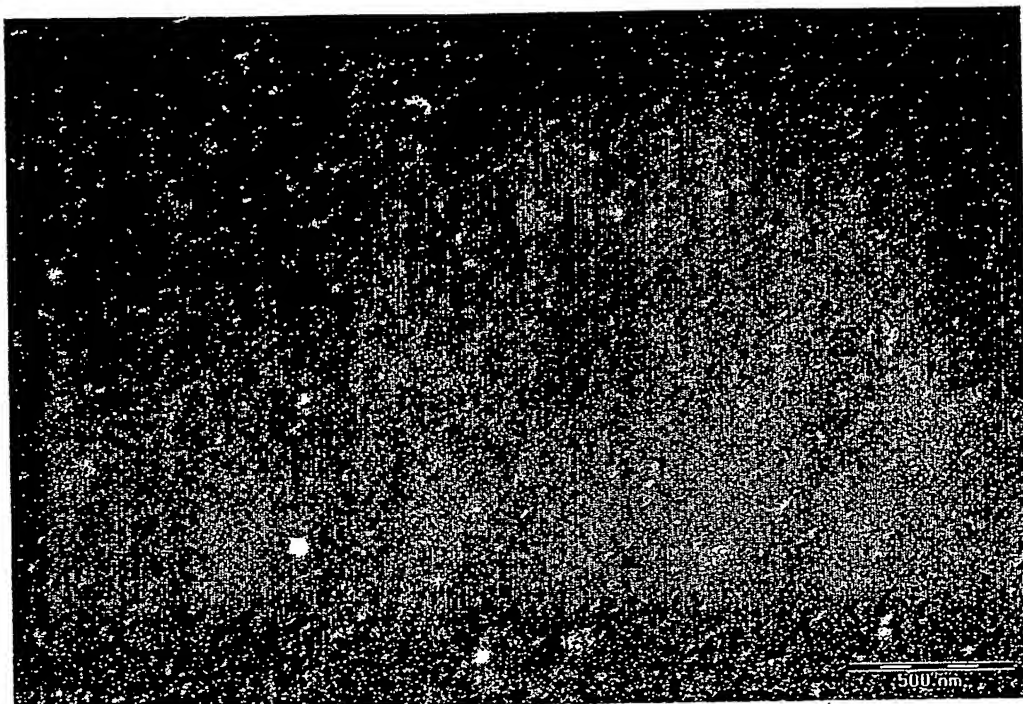
FIG 6



**FIG 7**



FIG 8A



**FIG 8B**